

Detection and Reduction of Microaggregates in Insulin Preparations

Caryn L. Heldt,¹ Mirco Sorci,¹ David Posada,² Amir Hirsra,² Georges Belfort¹

¹Howard P. Isermann Department of Chemical Engineering and The Center of Biotechnology and Interdisciplinary Studies, 110 8th Street, Troy, New York 12180, USA; telephone: 518-276-6948; fax: 518-276-4030; e-mail: belfog@rpi.edu

²Department of Mechanical, Aerospace, and Nuclear Engineering, Rensselaer Polytechnic Institute, Troy, New York

Received 19 April 2010; revision received 23 June 2010; accepted 27 July 2010

Published online 17 August 2010 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.22902

ABSTRACT: Insulin is an important biotherapeutic protein, and it is also a model protein used to study amyloid diseases, such as Alzheimer's and Parkinson's. The preparation of the protein can lead to small amounts of aggregate in the solution, which in turn may lead to irreproducible in vitro results. Using several pre-treatment methods, we have determined that pH cycling and diafiltration of the insulin removes microaggregates that may be present in the solution. These microaggregates were not detectable with traditional biochemical methods, but using small-angle neutron scattering, we were able to show that pH cycling reduces the radius of gyration of the insulin. Diafiltration removes the aggregates by size and pH cycling dissolves the aggregates by adjusting the pH through the pI of the protein. Pre-treating the insulin with either pH cycling or diafiltration allowed reproducible kinetics of fibrillation for the insulin protein. Microaggregates are a common problem in protein production, formulation, and preparation; here we show that they are the main cause for inconsistent behavior and how pH cycling and diafiltration can mitigate this problem.

Biotechnol. Bioeng. 2011;108: 237–241.

© 2010 Wiley Periodicals, Inc.

KEYWORDS: insulin; amyloid; microaggregates; diafiltration; pH cycling

The self-assembly of protein molecules into fibrils is associated with a group of diseases known as amyloid diseases, including Alzheimer's and Parkinson's. The cause of these diseases is not yet known, but it is expected that the trigger of the amyloid fibril formation may also be involved in the disease progression (Lansbury and Lashuel, 2006).

Correspondence to: G. Belfort

Contract grant sponsor: DOE

Contract grant number: DE-FE02-90ER14114; DE-FE02001ER46429

Contract grant sponsor: NSF

Contract grant number: CTS-94-00610

Additional supporting information may be found in the online version of this article.

The different proteins and peptides that are involved with various amyloid diseases vary in sequence, structure, and size. Yet, they all have a characteristic sigmoidal, nucleation-dependent kinetic curve consisting of (i) a lag-time, where nuclei are suspected to be formed, (ii) an elongation period where oligomers add to the nuclei to make fibrils, and (iii) an asymptotic period where most of the protein in solution has been depleted and elongation slows or stops. We have chosen to use insulin to study the kinetic progression of amyloid formation (Nayak et al., 2009a,b; Sorci et al., 2009). Insulin is a small, well-folded hormone that forms typical, long, narrow amyloid fibrils. It is a widely studied in vitro amyloid system and is implicated with a clinical syndrome, injection-localized amyloidosis (Dische et al., 1988).

The preparation of protein samples for the amyloid fibrillation assay is known to be problematic (Manno et al., 2007). Amyloid-forming peptides that have no known tertiary structure, for example, amyloid beta (A β ; Usui et al., 2009) and the synthetic peptide YEHK (Higashiya et al., 2007), may use harsh denaturing agents (6 M guanidine HCl and 8 M urea, respectively) to remove aggregated peptide prior to performing experimental assays. Others have used hydrophobic organic compounds [hexafluoro isopropanol (HFIP) and dimethyl sulfoxide (DMSO)] (Stine et al., 2003) or high pH to dissolve the aggregates of A β (Fezoui et al., 2000). These treatments were not feasible for a well-folded amyloid protein like insulin. A different approach must be taken to retain the native protein prior to fibrillation assays. In this paper, we report the use of pH cycling and diafiltration to remove aggregates from insulin solutions. The insulin aggregates are difficult to detect with traditional biochemical assays, but using small-angle neutron scattering (SANS) we show here that after pH cycling, the average radius of gyration (R_g) of insulin samples was reduced to values closer to those predicted and previously measured (Nayak et al., 2009b).

Insulin aggregates into amyloid fibrils in a short period of time when subjected to harsh conditions (pH 1.6 at 65°C). In the past, our group has worked with a batch of insulin that was “seed free” due to a reproducible 3-h lag-time with only a 0.22 μm filtration (Nayak et al., 2009b). When a new batch of insulin was received and fibrillation was performed without pre-treatment (Fig. 1A), it was found to commence anywhere between 1 and 3.5 h when monitoring the absorbance at 600 nm. This variability led us to seek a pre-treatment method that gave reproducible, reliable, and consistent insulin fibrillation kinetics. As a first step, we attempted to detect the presence of a contaminant in the insulin solution with size exclusion chromatography, mass spectrometry, and gel electrophoresis (see SI). The use of these methods showed that the insulin was either a monomer or dimer, depending on solution conditions. No evidence of contamination was detected. We then sought pre-treatment methods that would allow the removal of either large or small contaminants, since no specific contaminant had been detected. Insulin was filtered with a 0.2 μm filter to remove large protein aggregates (Fig. 1A) and this led to a longer, but still variable lag-time for the fibrillation process. To remove small contaminants,

Table I. Lag-time of pre-treated samples.

Sample	Lag-time (h)
No pre-treatment insulin	1.5 \pm 1.1
0.2 μm filtration	1.9 \pm 0.8
Dialysis	2.5 \pm 0.8
Diafiltration	3.2 \pm 0.1
pH cycling	2.2 \pm 0.1

Standard deviations were calculated from three independent runs.

which could possibly be an excipient or small protein fragments, the insulin solution was dialyzed, which was also unsuccessful (Fig. 1A). We finally found two techniques that gave reproducible fibrillation kinetics: diafiltration and pH cycling (Fig. 1B). The lag-times of three independent runs were fit to an empirical sigmoidal curve (Nielsen et al., 2001) for each pre-treatment and the calculated lag-times are shown in Table I. The standard error was greatly reduced for the diafiltration and the pH cycling pre-treatment.

Diafiltration, a popular method for buffer exchange, removed small contaminants, if any existed, through filtration with a 3 kDa membrane and buffer replacement. Large contaminants were removed with a 100 kDa membrane, as was mentioned by Manno et al. (2007), although they also stated that this step was later found to be unnecessary. The pore size of 100 kDa polymeric membranes has been measured with scanning tunneling microscopy (Bessières et al., 1996). Bessières et al. found that the pores were ellipsoids with average dimensions of 31 nm \times 16 nm. This is a cross-sectional area of \sim 1,600 nm², which corresponds to an equivalent circular pore of diameter 40 nm. Thus, the size of the main contaminants in the insulin solution ranged from about 0.04 to 0.2 μm .

Next, pH cycling was used to determine if the contaminants were insulin microaggregates. This would explain the lack of other contaminants with analytical probing (see SI). It is known that protein aggregates form in many stages of manufacturing and formulation (Cromwell et al., 2006) and that changing the pH may change the aggregation state of a protein (Moore et al., 1999). The present insulin fibrillation assays are run at a pH of 1.6 and 65°C. Insulin is poorly soluble in buffer at its pI \sim 5.4 (Wintersteiner and Abramson, 1933). By cycling the insulin from pH 1.6, through the pI, where it precipitates, up to pH 8, where it is again soluble, the insulin molecules experience many different pH values that in turn allow the dissolution of the protein microaggregates. The return of the solution to a pH of 1.6 again passes through the pI and allows for more disaggregation of the protein into dimers, which is the known state of insulin at pH 1.6 (Nielsen et al., 2001). It has recently been reported that changing from pH 7 to pH 1 leads to a change in insulin conformational flexibility (Haas et al., 2009). At pH 2–5, insulin has an increase in flexibility as compared with either a higher or lower pH. This is due to the competition of three amino acids to form a salt bridge; A51 on the B chain, and G1 and Q4 on the A chain. At a pH below 2 or above 5, there is a stable but different salt bridge

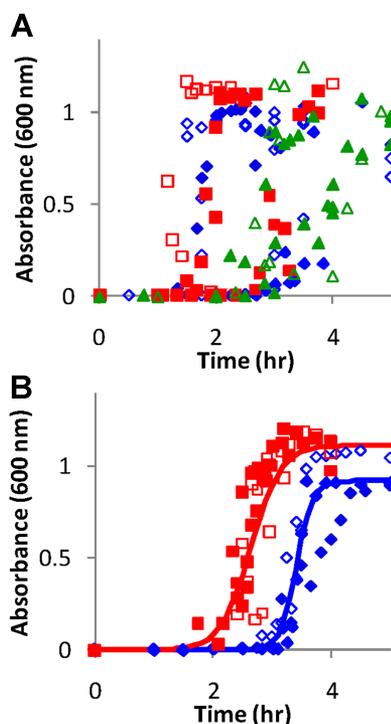


Figure 1. Insulin aggregation with different pre-treatment. **A:** With no pre-treatment (blue diamonds), filtration with a 0.22 μm filter (red squares), and dialysis with a 3.5 kDa cut-off membrane (green triangles) there was great variability in the time to form insulin fibrils. **B:** By using either pH cycling (red squares) or diafiltration (blue diamonds), consistency was found in the lag-time to form insulin fibrils. Lines were fit from an empirical model (Nielsen et al., 2001). All fibrillation assays were run at 2 mg/mL insulin in 100 mM NaCl at pH 1.6 and 65°C. The solid, open, and half filled symbols represent separate experimental runs. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

formed, so the reduced flexibility state has different conformations, indicating why insulin can form fibrils below a pH of 2, but not above a pH of 5 (Haas et al., 2009).

The results of aggregation assays with pH-cycled insulin are shown in Figure 1B. The lag-time is less than the diafiltered insulin, but reproducible results were obtained. The reproducibility of pH cycling demonstrates that the contaminants that were removed with diafiltration were most likely insulin microaggregates; by changing the pH and solubility of insulin, reproducible lag-times were obtained. If the contaminant was a molecule other than insulin, pH cycling could not have removed it and the lag-time would not have become consistent.

The lag-time of the pH-cycled insulin is notably less than the diafiltered insulin. The lag-time of the diafiltered insulin at 3.2 ± 0.1 h is close to other reported lag-times for insulin at similar experimental conditions (Nayak et al., 2009a; Nielsen et al., 2001). However, pre-treatment by diafiltration is time consuming, expensive (the regenerated cellulose membranes degrade at pH 1.6 with 10 diavolumes for a total of 8 h at 30 psi transmembrane pressure), and is difficult to control. Diafiltration removes more aggregates than pH cycling because it is a physical barrier method compared to a chemical method. Barrier methods are known to be more robust than chemical methods in the removal and purification of proteins. We determined that the lag-time for pH-cycled insulin is reproducible and provides useful information on the kinetics of the insulin fibrillation reaction. The pre-treatment time is shorter and pH cycling is easier and less expensive than diafiltration.

Looking at the data in Figure 1B, one notices that the post-diafiltered lag-time is, within error, the same as that reported by previous researchers (Nayak et al., 2009a; Nielsen et al., 2001; Sorci et al., 2009). We therefore conclude that most, if not all, of the microaggregates have been removed with this method. With pH cycling, however, this later conclusion cannot be made, especially since the lag-time remains shorter than expected. For this reason, SANS measurements were made on insulin without pre-treatment and post-pH cycling to measure the average size of the structures in solution (Fig. 2). The reduced data shown in Figure 2A was analyzed with the Guinier analysis (Fig. 2B and C and SI). The Guinier analysis gives an average R_g and mean molecular weight (MW) of the structures present in solution and the results are presented in Table II. The pH cycling of the insulin reduced the measured average R_g and MW to values much closer to theory than the ones obtained for untreated insulin. Although the average MW of the pH-cycled insulin was still above the theoretical value and the average R_g was below the theoretical value, the results obtained by SANS are a clear indication of the microaggregates existence in untreated insulin, and its consequent reduction after pH cycling.

The removal of microaggregates found in untreated insulin was incomplete for pH-cycled insulin, confirmed by the shorter lag-time of pH-cycled insulin as compared to diafiltered insulin (Fig. 1B) and by the SANS results showing

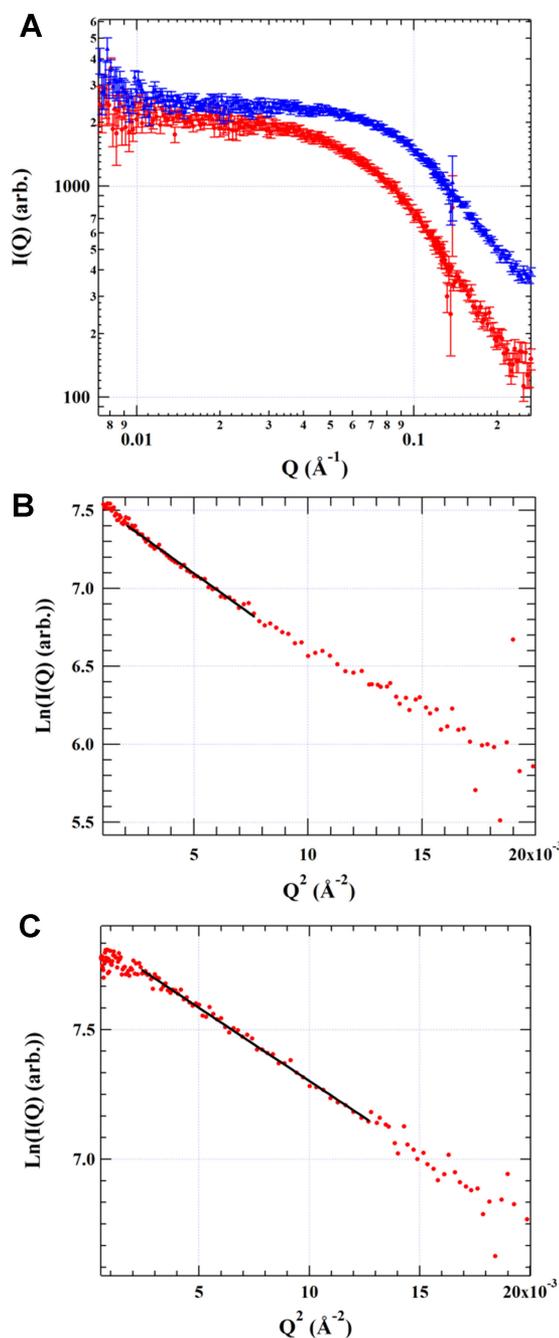


Figure 2. Small-angle neutron scattering (SANS) demonstrating a size difference between insulin with no treatment and pH cycling. (A) Reduced data, (B) Guinier analysis of no treatment insulin, and (C) Guinier analysis of pH-cycled insulin. All samples were at room temperature and the detector to sample distance was 1.7 and 6 m. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

that pH-cycled insulin did not have the R_g and MW theoretically predicted for insulin (Table II). One possible explanation is that one pH cycle dissolves large aggregates and leaves many small aggregates dispersed in the solution. The dispersion of microaggregates is more uniform and

Table II. Molecular weight and radius of gyration of samples studied with SANS*.

Sample	Molecular weight (Da)	Radius of gyration (Å)	$Q \times R_{\min}$	$Q \times R_{\max}$
No pre-treatment insulin	24,700 ± 300	18.3 ± 0.3	0.6	1.5
pH-cycled insulin	15,400 ± 100	12.7 ± 0.1	0.4	1.5
Insulin in D ₂ O ^a	10,820 ± 50	15.7 ± 0.2		
Theoretical insulin ^{a,b}	11,620	14.4		

*For the Guinier analysis to be valid, $Q \times R \approx 1$.

^aNayak et al., 2009b.

The values of MW and R_g for the theoretical insulin were from the formula weight and the molecular structure of insulin at pH 2, respectively.

allows for reproducible kinetics. Multiple pH cycles appear to remove even the smallest microaggregates and increase the lag-time to the expected value. Other possibilities are that the pH-cycled insulin has a different secondary structure or that different amino acids are solvent exposed. We examined the secondary structure change with circular dichroism (Fig. S4), and no change could be detected. It is known that amino acids of insulin rearrange in different pH conditions (Haas et al., 2009), but if these changes are permanent or transient, this is not known.

We hypothesize that there were non-covalent bound protein aggregates in insulin samples when the insulin was dissolved directly into buffer. The source of these aggregates is unknown. They could have come from manufacturing of the protein, the drying process, shipping or storage (excipient) conditions, or the buffer ionic strength and pH. They most likely arise from interprotein hydrophobic interactions. It has been shown that hydrophobic surface chemistry increases the rate of insulin aggregation and fibril formation (Nayak et al., 2008) and that hydrophobic lipids are associated with amyloid plaques formed in vivo (Gellermann et al., 2005). Recently, toxic oligomers of amyloid formation have been shown to have more solvent exposed hydrophobic patches than non-toxic oligomers (Campioni et al., 2010). These studies and others lead to the idea that protein aggregation is mediated by hydrophobic interactions. By cycling the protein through the pI, and therefore bringing the protein out of solution, and then returning it to a pH where it is soluble, a large percentage of the non-covalent bound aggregates are broken apart (re-dissolved) to create a more uniform solution. This uniform solution gives reproducible kinetics during insulin fibrillation that can be used as a model of amyloid formation in other proteins. We attempted to identify the entity that was causing irreproducible amyloid fibrillation kinetics and were able to detect a change in the average size of particles in solution with SANS. This and reproducible lag-times demonstrated that pH cycling and diafiltration were able to remove large aggregates in solution.

The method of pH cycling to remove aggregates in solution could apply to other proteins. In the Alzheimer's amyloid case, A β preparation at high pH has been shown to create seed-free preparations (Fezoui et al., 2000). It is also known that the aggregation of monoclonal antibodies is pH dependent (Perico et al., 2009). This work further

explored the use of pH that can be used to disaggregate proteins.

Materials and Methods

Human recombinant insulin was a gift from Novo Nordisk A/S, Denmark. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Insulin Fibrillation

The standard insulin fibrillation assay was performed as described earlier using 2 mg/ml of insulin in insulin buffer (100 mM NaCl and 25 mM HCl, pH 1.6) and heated to 65°C (Sorci et al., 2009).

Insulin Pre-Treatment

Different pre-treatments were used to find a reproducible fibrillation kinetic lag-time. One pre-treatment was filtration with a 0.2 μ m syringe filter made of cellulose acetate (Nalgene, Rochester, NY) at 10 mg/mL insulin. Another pre-treatment was dialysis with a Spectra/Por 3.5 kDa cut off membrane (Spectrum Laboratories, Rancho Dominguez, CA) of 10 mg/mL insulin dialyzed against insulin buffer at 4°C for 1–3 days.

Diafiltration was performed in an Amicon 10 mL dead-end filtration cell (Millipore Corporation, Lexington MA) equipped with a solvent reservoir for diafiltration. 25 mm diameter regenerated cellulose filtration membranes of 3 and 100 kDa MW cut off were utilized (Millipore, Bedford, MA). Transmembrane pressure was maintained at 30 psi using compressed nitrogen. Insulin at 10 mg/mL was diafiltered with a 3 kDa membrane for a total of 10 diavolumes, then the volume was reduced to the original volume of the insulin sample. The membrane was changed to a 100 kDa membrane and the solution was filtered through the membrane and collected.

The pH cycling of insulin was performed by making a solution of 20 mg/mL insulin in a total of 2 mL of buffer. The solution was filtered through a 0.2 μ m filter as stated earlier. It was then pH-cycled by adding 1 M NaOH in 20 μ L quantities to a final pH of 8. Then insulin buffer was added in 1 mL quantities until the pH reached 2.

All solutions were diluted with insulin buffer to 2 mg/mL and the pH adjusted to 1.6.

SANS

Small-angle neutron scattering was performed on the CG-3 beam line at Oakridge National Laboratory. Samples were either directly dissolved or pre-treated with pH cycling and then diluted to 6 mg/mL insulin at pH 1.6 in D₂O. Scattering was done at room temperature with a 2 mm path length cell. Guinier analysis of the data was performed as described in the Supplementary Information.

We thank Arne Staby, Novo Nordisk A/S for the generous gift of insulin and Mike Phillips, Millipore Corporation for the gift of membranes. We also thank Alvaro Ingles, University of Granada, for helpful discussions, Stephen Evans for assistance with the size exclusion chromatography, Dmitry Zagorevski for assistance with the mass spectrometry, and Shuo Qian, Qui Zhang, and Sai Venkatesh Pingali at Oakridge National Laboratory for assistance with the SANS experiments. The SANS measurement conducted at Oak Ridge National Laboratory's High Flux Isotope Reactor was sponsored by the Scientific User Facilities Division, Office of Basic Energy Sciences, U. S. Department of Energy. Financial support from DOE (DE-FE02-90ER14114 and DE-FE02001ER46429) and NSF (CTS-94-00610) is appreciated.

References

- Bessières A, Meireles M, Coratger R, Beauvillain J, Sanchez V. 1996. Investigations of surface properties of polymeric membranes by near field microscopy. *J Membr Sci* 109(2):271–284.
- Campioni S, Mannini B, Zampagni M, Pensalfini A, Parrini C, Evangelisti E, Relini A, Stefani M, Dobson CM, Cecchi C, Chiti F. 2010. A causative link between the structure of aberrant protein oligomers and their toxicity. *Nat Chem Biol* 6(2):140–147.
- Cromwell MEM, Hilario E, Jacobson F. 2006. Protein aggregation and bioprocessing. *AAPS J* 8(3):E572–E579.
- Dische FE, Wernstedt C, Westermark GT, Westermark P, Pepys MB, Rennie JA, Gilbey SG, Watkins PJ. 1988. Insulin as an amyloid-fibril protein at sites of repeated insulin injections in a diabetic patient. *Diabetologia* 31(3):158–161.
- Fezoui Y, Hartley DM, Harper JD, Khurana R, Walsh DM, Condron MM, Selkoe DJ, Lansbury PT, Fink AL, Teplow DB. 2000. An improved method of preparing the amyloid beta-protein for fibrillogenesis and neurotoxicity experiments. *Amyloid* 7(3):166–178.
- Gellermann GP, Appel TR, Tannert A, Radestock A, Hortschansky P, Schroeckh V, Leisner C, Lutkepohl T, Shtrasburg S, Rocken C, Pras M, Linke RP, Diekmann S, Fandrich M. 2005. Raft lipids as common components of human extracellular amyloid fibrils. *Proc Natl Acad Sci USA* 102(18):6297–6302.
- Haas J, Vöhringer-Martinez E, Bögehold A, Matthes D, Hensen U, Pelah A, Abel B, Grubmüller H. 2009. Primary steps of pH-dependent insulin aggregation kinetics are governed by conformational flexibility. *Chembiochem* 10(11):1816–1822.
- Higashiya S, Topilina NI, Ngo SC, Zagorevskii D, Welch JT. 2007. Design and preparation of beta-sheet forming repetitive and block-copolymerized polypeptides. *Biomacromolecules* 8(5):1487–1497.
- Lansbury PT, Lashuel HA. 2006. A century-old debate on protein aggregation and neurodegeneration enters the clinic. *Nature* 443(7113):774–779.
- Manno M, Craparo EF, Podesta A, Bulone D, Carrotta R, Martorana V, Tiana G, San Biagio PL. 2007. Kinetics of different processes in human insulin amyloid formation. *J Mol Biol* 366:258–274.
- Moore JMR, Patapoff TW, Cromwell MEM. 1999. Kinetics and thermodynamics of dimer formation and dissociation for a recombinant humanized monoclonal antibody to vascular endothelial growth factor. *Biochemistry* 38(42):13960–13967.
- Nayak A, Dutta AK, Belfort G. 2008. Surface-enhanced nucleation of insulin amyloid fibrillation. *Biochem Biophys Res Commun* 369(2):303–307.
- Nayak A, Lee CC, McRae G, Belfort G. 2009a. Osmolyte controlled fibrillation kinetics of insulin: New insight into fibrillation using the preferential exclusion principle. *Biotechnol Prog* 25(5):1508–1514.
- Nayak A, Sorci M, Krueger S, Belfort G. 2009b. A universal pathway for amyloid nucleus and precursor formation for insulin. *Proteins* 74:556–565.
- Nielsen L, Khurana R, Coats A, Frokjaer S, Brange J, Vyas S, Uversky VN, Fink AL. 2001. Effect of environmental factors on the kinetics of insulin fibril formation: Elucidation of the molecular mechanism. *Biochemistry* 40(20):6036–6046.
- Perico N, Purtell J, Dillon TM, Ricci MS. 2009. Conformational implications of an inversed pH-dependent antibody aggregation. *J Pharm Sci* 98(9):3031–3042.
- Sorci M, Grassucci RA, Hahn I, Frank J, Belfort G. 2009. Time-dependent insulin oligomer reaction pathway prior to fibril formation: Cooling and seeding. *Proteins* 77:62–73.
- Stine WB, Dahlgren KN, Krafft GA, LaDu MJ. 2003. In vitro characterization of conditions for amyloid-beta peptide oligomerization and fibrillogenesis. *J Biol Chem* 278(13):11612–11622.
- Usui K, Hulleman JD, Paulsson JF, Siegel SJ, Powers ET, Kelly JW. 2009. Site-specific modification of Alzheimer's peptides by cholesterol oxidation products enhances aggregation energetics and neurotoxicity. *Proc Natl Acad Sci USA* 106(44):18563–18568.
- Wintersteiner O, Abramson H. 1933. The isoelectric point of insulin. Electrical properties of adsorbed and crystalline insulin. *J Biol Chem* 99:741–753.